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Isolation and Phylogenetic Analysis of *Xylella fastidiosa* from Its Invasive Alternative Host, Porcelain Berry

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Abstract. A strain of *Xylella fastidiosa* was isolated from an invasive alternative host species, porcelain berry. Its genetic relationship with strains isolated from a native alternative host, wild grape; a nonnative alternative host, mulberry; and other economically important hosts including cultivated grape, peach, plum, oak, maple and oleander was determined by using sequence analysis of the 16S–23S rRNA intergenic spacer region. Our phylogenetic analysis revealed that the porcelain berry strain is most closely related to the wild grape strain. These two strains are more closely related to the oak, peach, and plum strains than to the mulberry and oleander strains. They are separated from the maple and cultivated grape strains. Our data suggest that suppression of porcelain berry, wild grape, and mulberry in the vicinity of susceptible economically important hosts such as oak, peach, and plum may provide an important control measure for diseases caused by *X. fastidiosa.*

Xylella fastidiosa Wells et al. [17] is a fastidious, Gram-negative, xylem-inhabiting, and leafhopper-transmitted bacterium, affecting over 30 plant families including such economically important crops as grape, peach, plum, citrus, coffee, oak, maple, and oleander [7, 15]. Although all strains of *X. fastidiosa* are currently classified as a single species, they differ in nutritional fastidiousness, plant host range, and pathogenicity [5]. Mehta and Rosato [11] distinguished five groups of *X. fastidiosa* by using sequence analysis of the 16S-23S rRNA intergenic spacer region: the citrus and coffee group; the peach, plum and oak group; the maple and grape group; the oleander group; and the pear group. The 16S–23S spacer region showed a higher level of variation than the 16S rRNA, and has also been used successfully to differentiate strains of other bacteria [9, 18].

A number of plant species, both native and invasive, have been identified as alternative hosts of *X. fastidiosa* including the native wild grape (*Vitis* spp.) [2, 3, 6, 10]. The nonnative mulberry (*Morus alba*), although sometimes used as an ornamental, can also be considered as an alternative host of *X. fastidiosa* because it has established itself in abandoned fields, waste areas, and roadsides since it was introduced from Asia in colonial times. It is unknown, however, how *X. fastidiosa* strains from alternative hosts are related genetically and pathogenically to those from economically important hosts. Such information is urgently needed for the development of effective control strategies for diseases caused by *X. fastidiosa.*

Porcelain berry (*Ampelopsis brevipedunculata* (Maxim.) Trautv.) is a vigorous, deciduous, woody perennial vine of the grape family. It was introduced from northeast Asia in the 1870's and is still used as a horticultural plant because of its unusual fruits and overall hardiness. Once established in the wild, however, it can quickly overwhelm trees and shrubs and out-compete native species for sun, water, and nutrients, making it an undesirable invader in the United States. Although porcelain berry was previously mentioned as a host of *X. fastidiosa* [15], no detailed study has been reported. To better understand genetic relationships between *X. fastidiosa* strains from alternative and economically important hosts, and to begin to define reservoirs of inoculum in nature for *X. fastidiosa* strains affecting economic hosts, we isolated *X. fastidiosa* from porcelain berry showing symptoms of infection by *X. fastidiosa.* We also

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isolated *X. fastidiosa* from wild grape (*Vitis* spp.) and mulberry (*Morus alba*) that were in the immediate vicinity of the porcelain berry. We then established phylogenetic relationships among the porcelain berry, the wild grape, and the mulberry strains, as well as between the porcelain berry and other host strains by sequence analysis of the 16S–23S rRNA intergenic spacer region.

Materials and Methods

Collection of plant samples. Leaves and young stems of the porcelain berry, wild-grape, and mulberry showing symptoms similar to those caused by *X. fastidiosa* were collected from the National Park Service Daingerfield Island Nursery in Alexandria, VA. Samples were sealed in individual plastic bags and used on the same or the next day for bacterial isolation. They were stored at 4°C and used for ELISA within 1 week.

ELISA. Collected plant samples were tested for the presence of *X. fastidiosa* by ELISA using a PathoScreen kit according to the manufacturer's instructions (Agdia, Inc., Elkhart, IN), with the following modifications. Leaf petioles were removed with a surgical blade, and 0.1 g of the plant tissue was cut into fine pieces. The plant tissue was placed in a FastDNA lysing matrix tube (Qbiogene, Inc., Carlsbad, CA), and 1 ml of Agdia's general extraction buffer was added to the tube. The tube was then placed in FastPrep FP120 instrument (Qbiogene) and processed at speed 4 for 40 s to homogenize the plant tissue. ELISA plates were incubated overnight at 4°C and were washed eight times with 1x washing buffer. Plates were read after 30 min at 492 nm in a FLUOstar Galaxy microplate reader (BMG Labtechnologies, Inc., Durham, NC).

Bacterial isolation. Isolations of *X. fastidiosa* were made from 0.05 g of leaf petioles. The surface of the plant tissue was surface-sterilized in 70% ethanol for 1.5 min, and then in 2% sodium hypochlorite for 1.5 min, followed by three 1-min washes in sterile water. The petiole was aseptically cut into 1-mm pieces and soaked in 500 μL sterile water for 30 min in a 1.7-mL microcentrifuge tube. Sixty microliters of the undiluted and diluted (1:10 with water) bacterial suspension were spread on periwinkle wilt (PW) plates [1], respectively, and incubated in the dark at 28°C. Bacterial colonies visible after 7 days with morphology typical for *X. fastidiosa* were tested further for their identities by PCR and subsequent sequencing of the PCR product as described below. Pure cultures were obtained by transferring single colonies to fresh PW plates.

DNA extraction and PCR. *X. fastidiosa* DNA was extracted by using DNeasy tissue kit according to the manufacturer's instructions (Qiagen Inc, Valencia, CA). Two microliters of the DNA extract were used for each PCR reaction. Specific PCR detection of *X. fastidiosa* was performed by using primers 272-1-int (5′-CTGCACTTACCCAATGCATCG-3′) and 272-2-int (5′-GCCGCTTCGGAGAGAGCATTCCT-3′) under the conditions described by Pooler and Hartung [12]. The 16S-23S rRNA intergenic regions of the porcelain berry, wild grape, and mulberry strains of *X. fastidiosa* was amplified by PCR with primers G1 (5′-GAAGTCGTAACAAGG-3′) and L1 (5′-CAAGGCATCCACCGT-3′) [8] under the conditions described by Purcell et al. [13]. PCR products were analyzed by electrophoresis in 0.8% (wt/vol) agarose gels stained with ethidium bromide.

Purification, cloning, and sequencing of PCR products. The PCR products synthesized with primers 272-1-int and 272-2-int, and the amplified 16S-23S spacer regions were purified with the QIAquick gel extraction kit (Qiagen) and were cloned into the pDrive cloning vector with Qiagen's PCR cloning kit. Desired clones were purified by using Qiagen's QIAprep spin miniprep kit. Both strands of the inserts in the clones were sequenced by using M13 forward (−21) and M13 reverse primers, respectively. The sequencing was done at the Center for Biosystems Research, University of Maryland, MD, USA.

Sequence analysis. The 16S-23S spacer sequences of *X. fastidiosa* strains were aligned by using CLUSTAL W (slow) [16]. Their phylogenetic relationships were determined by using the MegAlign module in the Lasergene sequence analysis software developed by DNASTAR, Inc. (Madison, WI). *Xanthomonas campestris* was designated as the outgroup.

Results and Discussion

Symptoms on the collected porcelain berry included marginal necrosis of leaves and bare petiole attached to stem, similar to those observed in *X. fastidiosa*- infected leaves of cultivated grapes and wild grapes. When tested by ELISA, the porcelain berry sample reacted positively with the antibody specific for *X. fastidiosa*, with an OD_{492} reading of 2.239 as compared with 0.016 for a buffer check, indicating the presence of the bacterium. The same plant sample was used for bacterial isolation, and bacterial colonies characteristic of *X. fastidiosa* were observed 7 days after incubation on PW plates. When their DNA was PCR amplified with *X. fastidiosa*- specific primers 272-1-int and 272-2-int [12], a 472-bp product was detected and further confirmed to be the predicted *X. fastidiosa* product by sequencing and sequence analysis with the reported genomic sequences of *X. fastidiosa* (data not shown).

We also isolated *X. fastidiosa* from wild grape and mulberry in close vicinity of the porcelain berry in the nursery. These plants showed similar leaf necrosis/scorch symptoms, so that their DNA could be used for genetic comparison and are samples from a natural plant community.

The sequences of the 16S–23S spacer region of *X. fastidiosa* strains from porcelain berry, wild grape, and mulberry were determined from their PCR products amplified with primers G1 and L1 [8]. The sequences of the 16S–23S intergenic regions from these strains were deposited in GenBank (Table 1). The spacer sequences of all three strains contained tRNAs for alanine (nucleotides 144–219, with UGC as anticodon) and isoleucine (nucleotides 233–309, with GAU as anticodon), as found in those of other strains of *X. fastidiosa* [4, 11].

A phylogenetic tree was constructed (Fig. 1) based on the 16S–23S spacer sequences of the three *X. fastidiosa* strains from porcelain berry, wild grape, and mulberry sequenced in this study and nine other selected spacer sequences (Table 1) available in GenBank. The nine selected sequences represented strains isolated from different geographic locations, when available, in the

Table 1. GenBank accession numbers for 16S-23S spacer sequences of *Xylella fastidiosa* strains used in this study

Strain	Host	Origin	GenBank accession No.	Reference or source
Xylella fastidiosa				
PB-Va	Porcelain berry	Virginia	AY196793	This study
WG-Va	Wild grape	Virginia	AY196795	This study
Mul-Va	Mulberry	Virginia	AY196794	This study
Oak88-9	Oak	Florida	AF073210	[13]
Stucky	Oak	Georgia	AF073214	[4]
Maple	Maple	California	AF073219	[13]
Ann1	Oleander	California	AF073215	[13]
STL	Grape	California	AF073228	[13]
PD95-2	Grape	Florida	AF073220	[4]
R116V3	Grape	Georgia	AF073223	[4]
Plum2#4	Plum	Georgia	AF073209	[13]
4S3	Peach	Georgia	AF073208	[4]
Xanthomonas campestris				
LMG568			AF209755	[11]

Fig. 1. Phylogenetic tree generated by the MegAlign program based on 16S–23S rRNA intergenetic spacer sequences of *X. fastidiosa* strains, with *X. campestris* as the designated outgroup. GenBank accession numbers for the 16S–23S spacer sequences are given in Table 1. The bar represents 1% nucleotide substitutions.

United States from both agricultural hosts including cultivated grape, peach, and plum, and ornamental hosts including maple, oak, and oleander. *X. campestris* was used as an outgroup. Our phylogenetic tree revealed five major branches of *X. fastidiosa* strains: the porcelain berry and wild grape strains, the mulberry strain, the oak, peach and plum strains, the maple and grape strains, and the oleander strain. The last four groupings of *X. fastidiosa* strains correspond well to the groupings of these strains obtained by other researchers using different molecular techniques [4, 11, 14]. Our analysis also showed that the porcelain berry strain is more closely related to the wild grape strain, and the porcelain berry and wild grape strains are more closely related to the oak, peach, and plum strains than to the mulberry and oleander strains. They are separated from the maple and cultivated grape strains, although their spacer sequences have 98.2–98.4% identities.

Our study is the first to determine genetic relationships among *X. fastidiosa* strains from hosts in a defined ecosystem, and between strains from alternative and economic hosts. It is not surprising that the porcelain berry strain is most closely related to the wild grape strain, since their hosts belong to the same plant family, Vitaceae. They are also commonly found next to each other, and probably were infected from a common source. What is interesting is that the porcelain berry and wild grape strains are more closely related to the oak

strain than to the mulberry strain, isolated from a plant located in close proximity to the porcelain berry and wild grape plants. This indicates that diverse strains of *X. fastidiosa* coexist in a natural ecosystem. It is also interesting that the wild grape strain is separated from the cultivated grape strains, although their hosts also belong to the same plant family. Since only three alternative host strains from one geographic location were used in our phylogenetic analysis, genetic relationships between alternative and economically important host strains need to be tested further with more isolates representing more alternative hosts from wider geographic areas. This may present a challenge, though, since most of the alternative hosts are symptomless and therefore sampling for and isolation of *X. fastidiosa* from these plants may be problematic [3, 6, 10]. Cross-inoculation experiments are also needed to determine how these strains are related pathogenetically.

The role played by the porcelain berry, wild grape, and mulberry in the spread of *X. fastidiosa* in economically important hosts such as oak, peach, and plum is still unknown, but may be significant considering their genetic relationship based on our study. All three hosts are commonly found in open areas and on forest edges [15]. Porcelain berry is distributed along the East Coast from New England to North Carolina where bacterial leaf scorch of landscape trees including oak caused by *X. fastidiosa* is widespread and becoming more severe. Wild grape is also found throughout central and northeastern United States, with mulberry in the mid-Atlantic area. Our data suggest that suppression of porcelain berry, wild grape, and mulberry in the vicinity of susceptible hosts such as oak, peach and plum may provide an important control measure for diseases caused by *X. fastidiosa.*

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Literature Cited

1. Davis MJ, Raju BC, Brlansky RH, Lee RF, Timmer LW, Norris RC, McCoy RE (1983) Periwinkle wilt bacterium: Axenic culture, pathogenicity and relationships to other Gram-negative, xylem inhabiting bacteria. Phytopathology 73:1510–1515
2. Freitag JH (1951) Host range of Pierce's disease virus as determined by insect transmission. Phytopathology 41:920–934
3. Hartman J, Saffray D, Beale J (2001) Presence of *Xylella fastidiosa* in symptomless landscape hosts. Nursery and landscape program-research report. Kentucky Agricultural Experiment Station, University of Kentucky, pp 24–25
4. Hendson M, Purcell AH, Chen D, Smart C, Guilhabert M, Kirkpatrick B (2001) Genetic diversity of Pierce's disease strains and other pathotypes of *Xylella fastidiosa.* Appl Environ Microbiol 67:895–903
5. Hopkins DL (1989) *Xylella fastidiosa:* xylem-limited bacterial pathogen of plants. Annu Rev Phytopathol 27:271–290
6. Hopkins DL, Adlerz WC (1988) Natural hosts of *Xylella fastidiosa* in Florida. Plant Dis 72:429–431
7. Hopkins, DL, Purcell, AH (2002) *Xylella fastidiosa:* cause of Pierce's disease of grapevine and other emergent diseases. Plant Dis 86:1056–1066
8. Jensen MA, Webster JA, Straus N (1993) Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. Appl Environ Microbiol 59: 945–952
9. Leblond-Bourget N, Philippe H, Mangin I, Decaris B (1996) 16S rRNA and 16S to 23S internal transcribed spacer sequence analyses reveal inter- and intraspecific *Bifidobacterium* phylogeny. Int J Syst Bacteriol 46:102–111
10. McElrone AJ, Sherald JL, Pooler MR (1999) Identification of alternative hosts of *Xylella fastidiosa* in the Washington, D. C., area using nested polymerase chain reaction (PCR). J Arboricul 25:258–263
11. Mehta A, Rosato YB (2001) Phylogenetic relationships of *Xylella fastidiosa* strains from different hosts, based on 16S rDNA and 16S-23S intergenic spacer sequences. Int J Syst 51:311–318
12. Pooler MR, Hartung JS (1995) Specific PCR detection and identification of *Xylella fastidiosa* strains causing citrus variegated chlorosis. Curr Microbiol 31:377–381
13. Purcell AH, Saunders SR, Hendson M, Grebus ME, Henry MJ (1999) Causal role of *Xylella fastidiosa* in oleander leaf scorch disease. Phytopathology 89:53– 58
14. Qin X, Miranda VS, Machado MA, Lemos EGM, Hartung JS (2001) An evaluation of the genetic diversity of *Xylella fastidiosa* isolated from diseased citrus and coffee in San Paulo, Brazil. Phytopathology 91:599–605
15. Sherald JL (2001) *Xylella fastidiosa,* a bacterial pathogen of landscape trees. In: Ash CL (ed) Shade tree wilt diseases. Proc. Wilt Diseases of Shade Trees: A National Conference, Aug. 25–28, 1999. St. Paul, MN: APS Press, pp 191–202
16. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680
17. Wells JM, Raju BC, Hung H, Weisburg WG, Mandelco-Paul L, Brenner DJ (1987) *Xylella fastidiosa* gen. nov., sp. nov: Gram-negative, xylem-limited, fastidious plant bacteria related to *Xanthomonas* spp. Int J Syst Bacteriol 37:136–143
18. Yoon JH, Lee ST, Kim SB, Goodfellow M, Park YH (1997) Inter- and intraspecific genetic analysis of the genus *Sacharomonospora* with 16S to 23S ribosomal DNA (rDNA) and 23S to 5S rDNA internally transcribed spacer sequences. Int J Syst Bacteriol 47: 661–669